# A New Look at Old Mutants of T4 DNA Polymerase

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ABSTRACT

The DNA polymerase and nuclease activities of bacteriophage T4 DNA polymerase mutants are discussed in the context of the crystal structure of the closely related bacteriophage RB69 DNA polymerase.

IT is fitting that this issue of Genetics honoring Jan Drake, who has made so many outstanding contributions to understanding the role of T4 DNA polymerase in maintaining the accuracy of bacteriophage T4 DNA replication, should coincide with the recent report of the crystal structure of the closely related bacteriophage RB69 DNA polymerase. The structure of this polymerase, as reported in the elegant work from the laboratory of Tom Steitz (Wang *et al.* 1997), provides new insight into early studies of T4 DNA polymerase mutations.

T4 DNA polymerase is an excellent model for determining how DNA polymerases control fidelity because of the ease with which phage with mutations in the polymerase gene can be isolated and studied (reviewed in Reha-Krantz 1994). Following the demonstration that T4 DNA polymerase was encoded by gene 43 (de Waard *et al.* 1965), and that mutation frequencies in unlinked genes were increased after infection with phage with some polymerase mutations (Speyer *et al.* 1966), Jan and his colleagues provided convincing evidence for the then surprising discovery that other polymerase mutations decreased the frequencies of some types of mutations and thus acted as "antimutators" (Drake and Allen 1968; Drake *et al.* 1969). These classic papers provided the foundation for genetic and biochemical studies showing that replication fidelity was determined by the accuracy of nucleotide selection during polymerization and by a balance between polymerization and excision by the 3′ to 5′ "proofreading" exonuclease activity of the polymerase (reviewed in Drake and Ripley 1994; Reha-Krantz 1994; and Goodman *et al.* 1993).

**Amber mutants:** My long and pleasant collaboration with Jan began with my unexpected finding that an amber mutant for the T4 polymerase gene (*amB22*) appeared to retain the exonuclease but not the polymerase activity of the full length protein. When I contacted Jan to ask for other gene 43 amber mutants, he was generous in sharing both his extensive mutant collection and his knowledge of their properties. He was properly skeptical about my observation, understood from my questions that I was not a geneticist, and gave me detailed instructions for backcrossing the mutant against the wild type to eliminate the possibility of additional mutations. When he wrote to thank me for the preprint of my "very biochemical paper" (Nossal 1969), I suspected this might not be a compliment. This paper showed that the exonuclease activity was retained by the amB22 and closely linked amC125 mutant proteins but not by the truncated proteins produced after infection with other amber mutations in gene 43. This was puzzling because Jan's mapping data indicated that he had sent me at least one mutation that should have given an amber fragment larger than that from *amB22*. [Jan's data established the order of the amber mutations within gene 43, but the direction of translation was determined later by Huang and Lehman (1972).]

The crystal structure of the RB69 DNA polymerase (Wang *et al.* 1997), provides a plausible explanation for why the amB22 fragment [731 amino acids (Reha-Krantz 1994)] retains the exonuclease but the longer amE4302 fragment (844 residues) does not. The RB69 polymerase (903 amino acids) is 62% identical to that of T4 (898 aa), and 14% of the remaining residues of the two proteins are chemically similar (Wang *et al.* 1995). Table 1 lists the amino acids altered in the mutant T4 polymerases discussed in this paper and those of their RB69 equivalents. The approximate positions of these mutations on the ribbon diagram of the RB69 polymerase are shown in Figure 1. Like other DNA polymerases of known structure (reviewed in Joyce and Steitz 1994), RB69 polymerase is organized into an exo domain with the active site for the proofreading exonuclease, a fingers domain thought to be involved in binding dNTP, a palm domain with the acidic residues essential for polymerization, and a thumb domain postulated to bind the duplex region of the primer-template (Figure 1).

The *amB22* and adjacent *amC125* mutations are located in the turn [between strand 25 and helix T (Wang *et al.* 1997)] that separates the palm from the C-terminal thumb. The nearly "thumb less" amB22 fragment is a stable soluble protein that was purified to apparent homogeneity (Nossal and Hershfield 1971). Its $V_{max}$ for

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**TABLE 1**

**Amino acids altered in T4 DNA polymerase mutants and the corresponding RB69 DNA polymerase residues**

| T4 mutant | T4 amino acid changed | RB69 amino acid |
|---|---|---|
| tsL56 | A89T and D363N | A91 and D366 |
| tsL98 | S345P | G348 |
| amE4322 | Q386am | Q389 |
| tsL88 | G694S | G697 |
| amC125 | Q730am | Q733 |
| amB22 | Q731am | K734 |
| tsL141 (tsCB120) | A737V | A740 |
| tsL141 supressor | L771F | L774 |
| tsL42 | A777V | A780 |
| amE4302 | W844am | W848 |

See Reha-Krantz (1994) for references for the isolation and sequencing of the T4 mutants and Wang *et al.* (1995) for the sequence of the RB69 DNA polymerase.

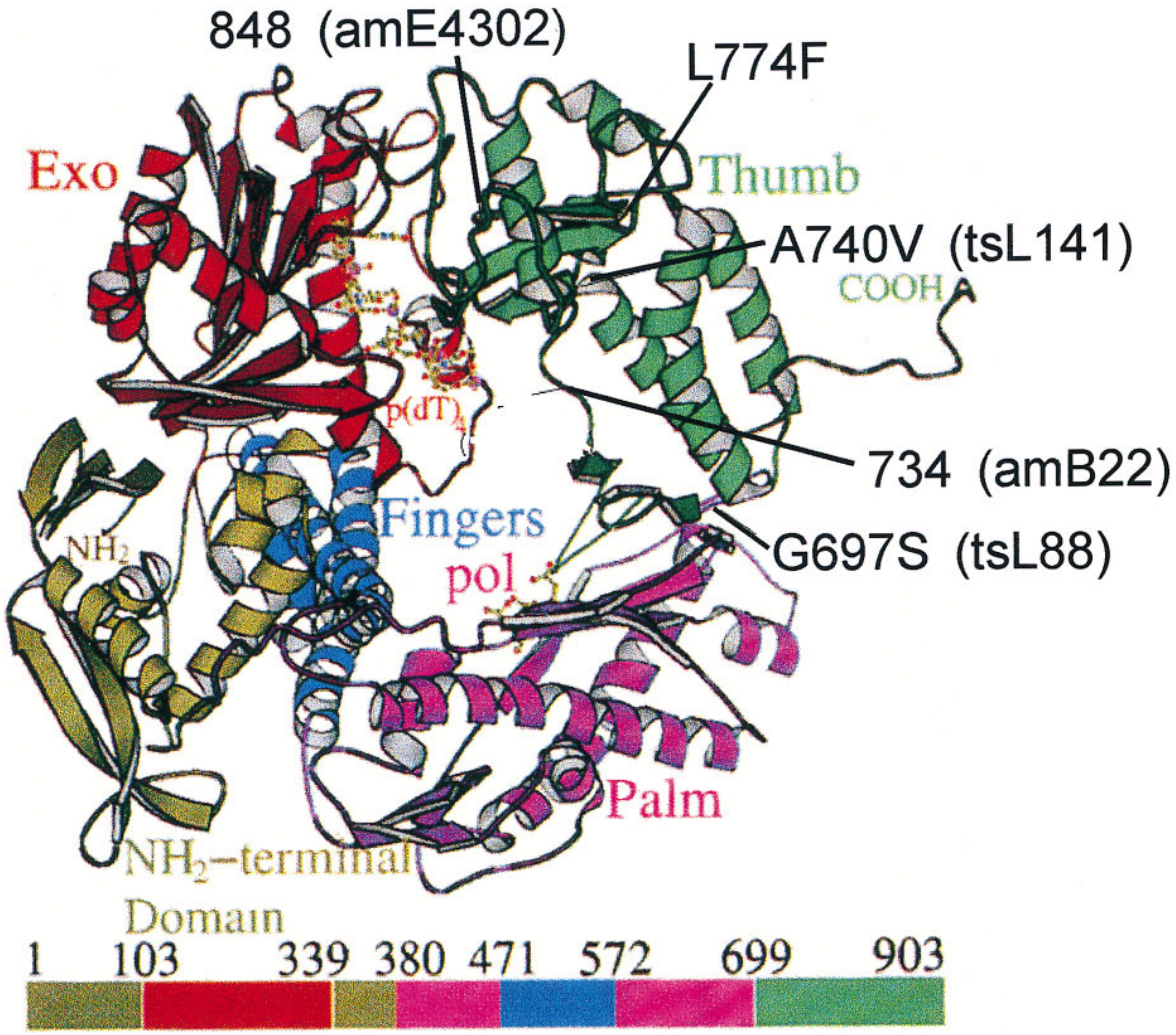


Figure 1.—Approximate positions on the ribbon representation of the RB69 DNA polymerase of residues corresponding to some of the T4 DNA polymerase mutations discussed in the text. The bar graph shows the residue numbers for the amino acids present in the polymerase domain with the same color: N-terminal domain, 1–103 and 339–380; exonuclease, 103–339; palm 380–471 and 572–699; fingers, 471–572; and thumb, 699–903. This figure is adapted from Wang *et al.* (1997), with permission from the authors. See Table 1 for the amino acids changed in other T4 DNA polymerase mutants.

the hydrolysis of single-stranded DNA or oligonucleotides ($n = 7$) was within twofold that of the wild-type enzyme. However, its apparent $K_m$ for each substrate was 10-fold higher, supporting the proposal (Wang *et al.* 1997) that the thumb of the RB69 type polymerases also helps to position the single-stranded DNA in the exonuclease active site. A T4 DNA polymerase fragment (1-388) containing the exonuclease domain without the palm, fingers, or thumb has been crystallized (Wang *et al.* 1996). The size of this fragment is close to that predicted for amE4322 which ends at 386 (Reha-Krantz 1994). However, detecting the residual nuclease activity in the 1-388 fragment (Lin *et al.* 1987) required protein concentrations much higher than those present in the fractions isolated from extracts of cells infected with the T4 amber mutants (Nossal 1969). Although all of the palm residues are present in the amB22 fragment, there was no evidence for even a single nucleotide addition at very high concentrations of dNTP and DNA template. This may reflect either the essential role of the thumb in positioning the primer for elongation, or disorder in the palm in the absence of the thumb region. T4 phage with the *amB22* mutation gave good phage production in suppressor strains that insert serine, glutamine, tyrosine and lysine (Reha-Krantz 1988), indicating that substitutions are tolerated in the turn separating the palm and thumb regions. However, all of these substitutions except glutamine gave phage with an antimutator phenotype.

The amE4302 mutant polymerase is truncated at the C-terminal end of strand 28, which is in the part of the thumb that lies closest to the exonuclease active site in the RB69 polymerase structure (Wang *et al.* 1997) (Figure 1). It seems likely that disorder in this region would interfere with the exonuclease activity. This amber mutation is not suppressed by strains that insert glutamine or lysine in place of the tryptophan present in the native T4 and RB69 enzymes (Reha-Krantz 1988). The possibility that the lack of nuclease in extracts of cells infected with T4 amE4302 was due to degradation or insolubility of the truncated protein was not investigated in these early studies.

After numerous phone calls, letters, and phage packages, I finally met Jan when he came to Washington for a meeting in the spring of 1969. I am sure of the date because Jan could not hide his surprise at finding that I was so close to becoming a mother. I reassured him that I was already balancing life in the lab and a young family. On subsequent visits he met the kids, and gained quite a reputation as a bedtime story teller.

**Mutators and antimutators:** Jan generously agreed to share his collection of temperature-sensitive mutator and antimutator polymerase mutants. With his guidance, we chose the T4 mutator *tsL88* and the antimutator *tsCB120* (identical to tsL141) for our biochemical studies of polymerase accuracy. The *tsL88* mutation lies just before strand 23, in the part of the thumb adjacent to the palm, within a block of residues highly conserved between T4 and RB69 but not present in other members of this polymerase family (Wang *et al.* 1997). This location is distant from the exonuclease domain, explaining why the tsL88 nuclease activity was similar to that of the wild type (Hershfield 1973). In contrast, many T4 mutator polymerases located in the exonuclease domain, such as the tsL98 and tsL56 enzymes studied by Bessman and his coworkers (Muzyczka *et al.* 1972), have exonuclease:polymerase ratios that are significantly lower than that of the wild type (reviewed in Reha-Krantz 1994). Michael Hershfield, then a postdoctoral fellow in my laboratory, used poly (dA) · (dT) and poly (dI) · (dC) primer-templates to show that the tsL88 mutator polymerase incorporated more incorrect nucleotides than the wild type, particularly at temperatures (30–34°) just below that at which this temperature sensitive polymerase was inactivated (Hershfield 1973). The *tsL88* mutation is within the cleft (D) that is postulated to hold the DNA duplex (Wang *et al.* 1997), and its apparent "$K_m$" for poly (dA) · (dT) was 100-fold higher than that of the wild type (Gillin and Nossal 1976a). The dNTP binding site on T4 DNA polymerase must include the DNA template, since the enzyme has a much higher affinity for complementary than noncomplementary dNTP (Gillin and Nossal 1975) (Capson *et al.* 1992). With saturating template concentrations, there was no significant difference between the wild-type and tsL88 polymerases in their affinity for correct or incorrect dNTP (Gillin and Nossal 1976a).

We wanted to work with the tsCB120 (A737V) antimutator polymerase because Jan and his colleagues had extensively characterized the spectrum of mutations whose frequency was decreased by this mutation (Drake and Allen 1968; Drake *et al.* 1969). We found that the exonuclease activity of the purified tsCB120 enzyme excised a much larger proportion of the newly incorporated nucleotides than the wild-type enzyme (Gillin and Nossal 1976a), in agreement with the observations of the Bessman laboratory with the tsL141 and the nearby (A777V) tsL42 antimutator enzymes (Muzyczka *et al.* 1972). This increased turnover of newly incorporated nucleotides was not due to a hyperactive nuclease, because the A737V polymerase had 3′ to 5′ exonuclease activity on single-stranded DNA substrates that was no higher than that of the wild type (Gillin and Nossal 1975; Spacciapoli and Nossal 1994b). Our more recent studies show that this single mutation increases the processivity of the enzyme as a nuclease but decreases its processivity as a polymerase (Spacciapoli and Nossal 1994b).

The A737V polymerase is also defective in strand displacement synthesis. The polymerase alone had difficulty copying templates like poly d(AT) in which primer extension requires displacement of nucleotides paired with the template (Gillin and Nossal 1976b). In contrast to the wild-type polymerase, the A737V mutant enzyme was unable to copy duplex DNA templates in a replication system with the T4 polymerase accessory proteins, single-stranded DNA binding protein, and the

gene 41 helicase (Spacciapoli and Nossal 1994a). The T4 gene 59 helicase assembly protein, which helps to load the helicase at the replication fork, was essential for strand displacement synthesis by the A737V polymerase. We have suggested that the increased nucleotide turnover and defective strand displacement synthesis are related manifestations of abnormal partitioning of the primer terminus between the polymerase and exonuclease active sites on the A737V enzyme (Spacciapoli and Nossal 1994a,b). Our experiments also suggest that the A737V polymerase does not couple effectively with the helicase. Whether this results from a change in the protein surface that interacts with the helicase or the slow rate of translocation by the mutant polymerase remains to be determined.

Linda Reha-Kranz and her colleagues identified intragenic suppressors of the A737V polymerase by screening for second-site mutations that restored the ability of the mutant phage to grow on *E. coli OptA1* and thus were assumed to have decreased nucleotide turnover by the A737V polymerase (Stocki *et al.* 1995). We have shown that one of these second-site mutations (L771F) partially restores the polymerase processivity and strand displacement synthesis *in vitro* (Spacciapoli and Nossal 1994a,b). The RB69 polymerase structure shows that T4 A737 and L771 are located close to each other within the thumb region. RB69 A740 is near the N-terminal end of helix T and RB69 L774 is just after the C-terminal end of helix U (Figure 1) (Wang *et al.* 1997). Mutation of T4 L771 to histidine also suppressed the A737V mutation, as did insertion of valine or aspartic acid following L771 (Stocki *et al.* 1995). Thus L771 appears to be a hotspot for suppressor mutations. Wang *et al.* (1997) were able to visualize four residues of oligo (dT) bound in the exonuclease-active site of the RB69 DNA polymerase. S735 leading into helix T is close to the fourth nucleotide from the 3′ end. Thus it is plausible that substituting a larger valine residue in place of T4 A737, at the top of helix T, would affect the channel crossed by the primer terminus as it switches between the polymerase and exonuclease active sites. A clearer understanding of the interaction between T4 A737 and L771, and their relationship to the primer terminus, must await the solution of the structure of a cocrystal of the polymerase and a primer-template.

I thank Tom Steitz for permission to use the ribbon diagram of the RB69 DNA polymerase.

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